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# Experimental and Clinical Homotransplantation of the Aortic Valve; Antigenicity of Canine and Human Aortic Valves Demonstrated by Fluorescent Antibody Techniques

by

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Prosthetic valves have been used to replace diseased aortic valves for the past several years since the first successful case reported by HUFNAGEL in 1952<sup>1)</sup>. However, in spite of remarkable recent progress, the disadvantages of aortic ball valves, such as thromboembolism, ball variance, and hydraulic inefficiency, have not yet been completely overcome.

Success with an aortic valve homograft in the descending aorta was reported by MURRAY<sup>2)</sup> in 1956, and since 1962 subcoronary transplantations of aortic valve homografts have been carried out by ROSS<sup>3)4)5)6)7)</sup> and BARRATT-BOYES<sup>8)9)10)11)12)</sup>. In their large series of cases of transplantation to the subcoronary position, these two pioneer surgeons have shown that (1) patients with homografts do not require postoperative anticoagulant therapy, (2) homograft aortic valves have excellent hydraulic efficiency, (3) this kind of operation can be performed with an acceptably low mortality rate and a lower incidence of infection than the insertion of a prosthetic valve, and (4) a homograft aortic valve will function satisfactorily for a long time without any significant immunological problems. Recently most cardiovascular surgeons have been using homografts rather than prosthetic valves to replace diseased aortic valves.

It is essential, however, to study in detail the antigenicity of the homograft aortic valve and the fate of transplanted homografts after various intervals, before the clinical use of homograft aortic valves can progress. Even the two pioneer surgeons, ROSS and BARRATT-BOYES, performed this operation without any basic experimental study, and no investigation of immunological properties or localization of the antigenic substances in the aortic valve has yet been reported in the literature.

The present studies on experimental homotransplantation of the aortic valve in mongrel dogs since 1964 show that although antigenic substances can be demonstrated in canine aortic valves by fluorescent antibody techniques, even fresh homograft aortic valves transplanted to the subcoronary position continue to function perfectly for up to 2 years without any immunological problems or loss of pliability of the leaflet. The gradual replacement of grafted valves by host tissues was also demonstrated by fluorescent antibody techniques and the usual histological methods.

Since October 1967, on the basis of these experimental results, homotransplantation of the aortic valve was performed in four patients with confidence that the grafts would remain functional for long periods of time.

## PART I. STUDY OF THE ANTIGENICITY OF CANINE AND HUMAN AORTIC VALVES BY FLUORESCENT ANTIBODY TECHNIQUES

### A. Introduction

In about 25% of adults aged 20 to 40 years<sup>13)</sup>, the aortic cusps are avascular except at the immediate area of attachment. It would be expected, therefore, by analogy with corneal homotransplantation, that the immunological problems arising from clinical homotransplantation of the aortic valve would not be too great. MOHRI et al.<sup>14)</sup> found in their experimental studies on dogs that the prior sensitization of the dog by skin grafting from the same donor did not alter the fate of fresh homologous aortic valves transplanted to the subcoronary position.

In this study, the immunological properties and distribution of the antigenic substances in canine and human aortic valves were investigated by fluorescent antibody techniques.

### B. Materials and Methods

#### 1. Preparation of the Antisera

Fresh aortic valves of healthy mongrel dogs were separated from the heart muscles and aortic wall and washed three times with physiological saline in different plates. Two hundred milligrams of valve tissue were homogenized in a POTTER blender in 1 ml. of physiological saline. This emulsion was mixed thoroughly with an equal volume of complete FREUND's adjuvant (Difco Laboratories Detroit, Mich.) and 1.5 to 2.0 ml. of this mixture was injected intramuscularly into albino rabbits weighing 2 to 2.5 kg. After five or six immunizations at intervals of two weeks, 2.0 ml. of this mixture was injected intramuscularly as a booster.

Anti-canine whole serum rabbit antiserum was prepared by the intramuscular injection into albino adult rabbits of 1 ml. of a mixture of equal volumes of canine whole serum and incomplete FREUND's adjuvant. About 4 weeks later, 0.2 ml. of canine whole serum was injected intravenously as a booster. All procedures were carried out aseptically.

Crystallized human serum albumin and gamma G-globulin obtained from Sigma Chemical Co. (St. Louis, Mo.) were each mixed thoroughly with an equal volume of incomplete FREUND's adjuvant (Difco). Ten to twenty milligrams of each antigen was injected intramuscularly and, about four weeks later, each fluid antigen in 10 mg doses was injected intravenously as a booster injection. In all cases, intravenous booster injections were followed by the intravenous injection of 5 mg. of 2-(benzhydryl-oxy)-N, N-dimethylethylamine hydrochloride (Benadryl).

All antisera were drawn 5 to 7 days after a booster injection.

The titers of these antisera were measured by the precipitin curve method. The nitrogen content was determined by the micro-Kjeldahl method in a modification of the method of KOCH and McMEekin<sup>15)</sup>.

#### 2. Preparation of Conjugates

Globulin fractions of each of the antisera were prepared by precipitating them three times with one third saturated ammonium sulfate.

The globulins of anti-canine aortic valve and anti-canine gamma globulin rabbit antiserum were conjugated with fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory, Inc., Baltimore, Md.) by the method of MARSHALL, EVELUND and SMITH<sup>16)</sup>, while the globulins of anti-canine whole serum and anti-human serum albumin rabbit antiserum were conjugated with tetramethylrhodamine isothiocyanate (Baltimore Biological Laboratory, Inc.). With FITC, the ratio of dye to protein was 1 : 40, and the reaction period was six hours at 4°C.; with rhodamine isothiocyanate, a dye to protein ratio of 1 : 20 was used and a reaction period of 18 hours at 4°C. Following separation from the free fluorescent dyes by gel filtration on Sephadex G-50 (Pharmacia, Uppsala), the conjugates were purified by fractionation on DEAE-cellulose (0.9 meq/g, Brown Company Berlin, N. H.) columns as described by McDEVITT et al.<sup>17)</sup> Fractions eluted at pH 6.4 with 0.05 M or 0.10 M phosphate buffer were used for staining. Each fraction was concentrated with ethylene glycol (mol wt, 6000) and dialyzed against several changes of isotonic phosphate-buffered saline (8 g. NaCl, 3.22 g. Na<sub>2</sub>HPO<sub>4</sub> 12H<sub>2</sub>O and 0.45 g. NaH<sub>2</sub>PO<sub>4</sub> 2H<sub>2</sub>O in 1 liter of distilled water, pH 7.2 ; phosphate-buffered saline).

### 3. Assays for Specificity of Antisera

Specificity of antibody was examined by the double gel diffusion method of OUCHTERLONY and by immunoelectrophoresis. Double diffusion studies were carried out on glass microscope slides layered with 1 % purified agar (Difco Laboratories) prepared in phosphate-buffered saline. A modification of HIRSCHFELD's technique<sup>18)</sup> was used for immunoelectrophoresis. Glass plates, 76 × 26 mm, were covered with a 1.2 mm. thick layer of 1 % purified agar prepared in pH 8.6 veronal-lactate buffer, ionic strength 0.05. Electrophoresis of various antigens was at 8 mA/plate and 120 V. for 45 minutes. After electrophoresis, the trench was filled with antiserum and incubated at room temperature overnight and then left standing in the cold for two to four days. Figs. 1 and 2 prove the specificity of the anti-human serum gamma globulin rabbit antiserum and anti-human serum albumin rabbit antiserum used in these studies.

### 4. Preparation of Sections

Fresh aortic valves of mongrel dogs, including some adjacent tissues, were excised from the beating heart. Human aortic valve was obtained within two hours after death. All specimens were washed three times with cold phosphate-buffered saline in order to remove the serum proteins adhering to the surface of the valves immediately after excision from the host, and were cut 2 mm. thick sections at right angles to the line of insertion of the cusp. Then they were fixed in cold 95 % ethanol and kept for about 24 hours at 4°C. and were dehydrated, cleared and paraffin-embedded as described by SAINTE-MARIE<sup>19)</sup>. Sections (3  $\mu$  to 4  $\mu$ ) were cut, deparaffinized with xylene and hydrated through successive ethanol baths.

### 5. Staining with Fluorescein-labeled Conjugates

After washing with phosphate-buffered saline, sections were stained with fluorescein-labeled conjugates in a moist chamber at room temperature for 16 hours. These slides were then washed with phosphate-buffered saline, mounted in glycerol containing 10 % phosphate-buffered saline and examined under a Carl Zeiss fluorescence microscope.

A double-staining method was used for the detection of valve-specific antigens in the canine aortic valve. The valves were stained by exposing them first to rhodamine-labeled

anti-canine whole serum antibody for 16 hours and then to FITC-labeled anti-canine aortic valve antibody for 16 hours.

### C. Results

#### 1. Canine Aortic Valve

##### (1) Detection of valve-specific antigens in canine aortic valves

Figs. 3 and 4 show the immunoelectrophoresis of the emulsion of canine aortic valve and canine whole serum. When the valve emulsion was electrophoresed and each trench was filled with either anti-canine aortic valve rabbit antiserum or anti-canine whole serum rabbit antiserum, the same precipitin patterns were observed against both of the antisera, as shown in Fig. 3. On the other hand, all precipitin arcs, which were formed between the normal canine whole serum and anti-canine aortic valve antiserum, corresponded to some of the precipitin arcs which were formed between the normal canine whole serum and anti-canine whole serum antiserum (Fig. 4). Some antigenic components of the canine serum proteins were revealed in the canine aortic valve. However, there was no valve-specific antigen in the canine aortic valve.

This finding was confirmed by the absorption test and the double staining method of the fluorescent antibody technique.

Absorption of anti-canine aortic valve rabbit antibodies with successively diluted volumes of canine whole serum was carried out in the cold for two days following storage for two hours at 37°C. By the double gel diffusion method of OUCHTERLONY, no precipitin band could be found between the emulsion of canine aortic valve and absorbed anti-canine aortic valve rabbit antisera (Fig. 5).

Canine aortic valves stained with both FITC-labeled anti-canine aortic valve and rhodamine-labeled anti-canine whole serum antibodies showed specific yellow fluorescence. Neither green nor orange-red fluorescence was observed separately in any fluorescent area of the leaflet.

##### (2) Localization of antigens in canine aortic valves

There was no difference in the locations of specific fluorescence of the sections stained with anti-canine aortic valve conjugate or anti-canine whole serum conjugate, as mentioned previously.

The most intense specific fluorescence was observed in the collagen fibers adjacent to the elastic fibers on the ventricular side of the leaflet (Fig. 6).

The endothelium on both ventricular and aortic sides of the leaflet, the thin layer of collagen fibers just beneath the endothelium of the aortic side of the leaflet (Fig. 6) and the fibrous tissues between the cardiac muscles and the base of the cusp also showed specific bright fluorescence.

However, for about one third of the thickness of the leaflet there was no specific fluorescences with either of the conjugates. The non-stained region was located between the thin layer of the fluorescent collagen fibers just beneath the endothelium of the aortic side of the leaflet and the broad intensely fluorescent part containing the elastic fibers on the left ventricular side of the leaflet (Fig. 6). This region was not stained at all at the base of the cusp, but it contained fibers which showed a specific faint fluorescence in areas from the middle to the apex of the leaflet. The apex of the leaflet contained mostly non-fluorescent tissues mixed with a few scattered faintly fluorescent fibers.

The elastic structures along both the ventricular and the aortic sides of the leaflet showed strong autofluorescence but were never stained with either of the conjugates.

### (3) Antigens in other valves of the canine heart

Sections of the mitral, tricuspid and pulmonary valves were examined by the same fixatives, fixation procedures and staining methods and were also found to stain brightly in some parts with rhodamine-labeled anti-canine whole serum or FITC-labeled anti-canine aortic valve rabbit antibody. Furthermore, all of the specifically fluorescent parts of these valves, which were stained by the double staining method, showed yellow, but not green or orange-red, fluorescence. These results indicate that all of the four canine cardiac valves (aortic, pulmonary, tricuspid and mitral) have common antigenic substances which are the same as some of the components of canine serum proteins.

### 2. Tissues of Canine Heart Adjacent to Aortic Valve

The sarcolemma of the cardiac muscle fibers was intensely stained with both anti-canine whole serum and anti-canine aortic valve conjugates, but the sarcoplasm of the cardiac muscle fibers was not stained at all (Fig. 7).

In the endocardium, strong fluorescence was found in the endothelium and in the subendothelial fibrous tissues. The connective tissue at the base of the cusp adjacent to cardiac muscles showed intense specific fluorescence with both conjugates.

### 3. Human Aortic Valve and Adjacent Tissues

Human aortic valves showed intense specific fluorescence with FITC-labeled anti-human serum gamma globulin rabbit antibody. Fluorescence, specific for gamma globulin, was observed in the same areas as in canine aortic valves stained with anti-canine aortic valve or anti-canine whole serum conjugate. These areas were (a) the collagen fibers adjacent to the elastic fibers on the ventricular side of the leaflet (Fig. 8), (b) the endothelium on both ventricular and aortic sides of the leaflet, (c) the thin layer of collagen fibers just beneath the endothelium of the aortic side of the leaflet, (d) the fibrous tissues between the cardiac muscles and the base of the cusp, and (e) the endothelium and sub-endothelial fibrous tissues of the endocardium.

However, the sarcolemma of human cardiac muscle fibers did not show specific fluorescence with anti-human serum gamma globulin conjugate.

Any fluorescence was too faint to be called specific for human serum albumin in the sections of the human aortic valve stained with rhodamine-labeled anti-human serum albumin rabbit antibody, even on the left ventricular side of the leaflet or in the fibrous tissues between the cardiac muscles and the base of the cusp (Fig. 9). The elastic structures on both sides of the human aortic valve showed strong autofluorescence, but never stained with anti-human gamma globulin or anti-human albumin conjugate.

No staining was demonstrated in any of the control sections of the canine or human aortic valves.

### *D. Discussion*

Thinner sections and all unimpaired parts of the leaflet in one section were obtained more easily from paraffin than from frozen blocks. Moreover, the antigenicities of tissue components and of serum proteins remained unchanged during the procedure of paraffin embedding<sup>19)21)</sup>. Therefore, fixation with 95 % ethanol and paraffin embedding were employed in these studies. In spite of the presence of albumin in canine aortic valves as

proved by immunoelectrophoresis, specific fluorescence for human serum albumin in human aortic valves was not demonstrated except for very faint fluorescence in some part of the valve. In order to elucidate whether or not human serum albumin is present as an antigen in human aortic valves, it may be necessary to use other fixatives and fixation procedures or more sensitive techniques.

Immunodiffusion, immunoelectrophoretic assay and fluorescent antibody techniques have shown that the antigenic substances in the canine aortic valve possess antigenicities similar to some canine serum proteins, such as albumin, gamma globulin, etc. However, it is not known whether the antigenic proteins in the canine aortic valve are the same as those in canine whole serum, or whether there are immunologic cross-reactivities between them.

Human and canine endothelium of the aortic valve and endocardium showed specific fluorescence when stained with anti-human serum gamma globulin, anti-canine aortic valve or anti-canine whole serum conjugate, in spite of adequate washing of the specimens with cold phosphate-buffered saline before fixation. It seems probable that the endothelium itself contains these antigenic proteins or that the serum proteins penetrate into the endothelium.

In their clinical case reports of homotransplantation of the aortic valve, DAVIES et al.<sup>6)</sup> and HUDSON<sup>20)</sup> stated that none of the various tests used disclosed a specific and significant immune reaction in the patients. But their patients had received nonviable grafts in which all the proteins had been denatured. On the other hand, in patients who receive transplants of fresh, viable grafts, sensitive methods might be expected to demonstrate some circulating antibodies in the serum. However, according to the results of the present study, it seems very difficult to demonstrate antibodies specific for the transplanted valve in the sera of patients who received a homologous aortic valve, because of the absence of tissue-specific antigen in the aortic valve and the "privileged site" of the subcoronary position.

#### *E. Summary*

1. No tissue-specific antigen could be demonstrated in canine aortic valves by immunodiffusion, immunoelectrophoresis or fluorescent antibody techniques.

2. Antigenic components of canine serum proteins were found in canine aortic valves.

3. All of the four canine cardiac valves contained antigenic substances common to some of the components of canine serum proteins.

4. The antigens in the canine aortic valve and tissues adjacent to the cusp were found in the following positions: (a) collagen fibers along the elastic fibers on the ventricular side of the leaflet, (b) thin layer of collagen fibers just beneath the endothelium of the aortic side of the leaflet, (c) endothelium of the endocardium and both sides of the leaflet, (d) fibrous tissues between the cardiac muscles and the base of the cusp, (e) sarcolemma of the cardiac muscle fibers, and (f) subendothelial fibrous tissues of the endocardium.

5. Fluorescence, specific for human serum gamma globulin, was observed in the same locations as the antigenic components of canine serum proteins on the canine aortic valve. However, human gamma-globulin-specific fluorescence could not be demonstrated in the sarcolemma of human cardiac muscle fibers.

6. Human aortic valves showed almost no staining with rhodamine-labeled anti-human serum albumin rabbit antibodies.



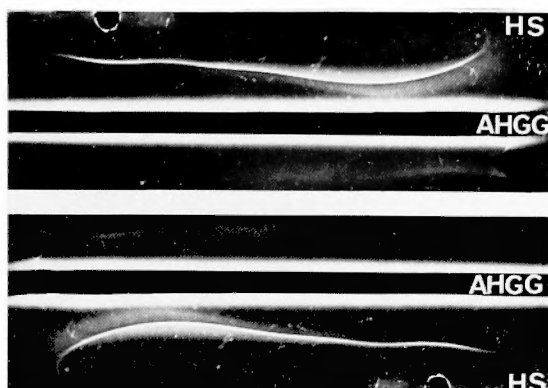


Fig. 1



Fig. 2

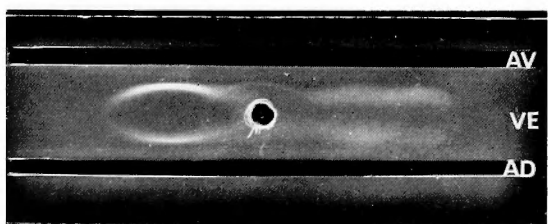


Fig. 3

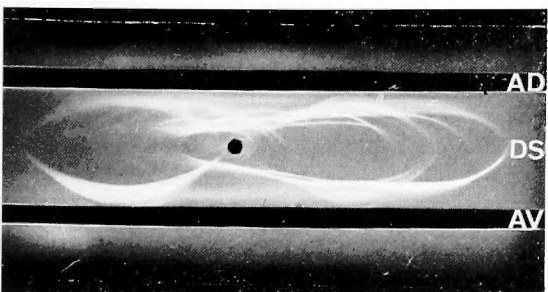


Fig. 4

**Fig. 1** Immunoelectrophoresis of normal human serum. Both upper and lower wells are filled with normal human serum. Only one precipitin arc is formed with each specimen of anti-human serum gamma globulin rabbit antiserum filling the trenches.

HS : normal human serum

AHGG : anti-human serum gamma globulin rabbit antiserum

**Fig. 2** Immunoelectrophoresis of normal human serum and human serum albumin used as an antigen for immunization. The upper well is filled with normal human serum, and the lower is filled with human serum albumin used as an antigen. Only one precipitin arc is formed between each antigen and anti-human serum albumin rabbit antiserum filling the trench.

HS : normal human serum

AL (Ag) : human serum albumin used as an antigen

AHAL : anti-human serum albumin rabbit antiserum

**Fig. 3** Immunoelectrophoresis of emulsion of canine aortic valve. The upper trench is filled with anti-canine aortic valve rabbit antiserum. The lower is filled with anti-canine whole serum rabbit antiserum. Emulsion of canine aortic valve forms the same precipitin pattern against both antisera.

AV : anti-canine aortic valve rabbit antiserum

VE : emulsion of canine aortic valve

AD : anti-canine whole serum rabbit antiserum

**Fig. 4** Immunoelectrophoresis of normal canine whole serum. The upper trench is filled with anti-canine whole serum rabbit antiserum. The lower is filled with anti-canine aortic valve rabbit antiserum. All precipitin arcs, which are formed between the normal canine whole serum and anti-canine aortic valve antiserum, correspond to some of the precipitin arcs which are formed between the normal canine whole serum and anti-canine whole serum antiserum.

AD : anti-canine whole serum rabbit antiserum

DS : canine whole serum

AV : anti-canine aortic valve rabbit antiserum



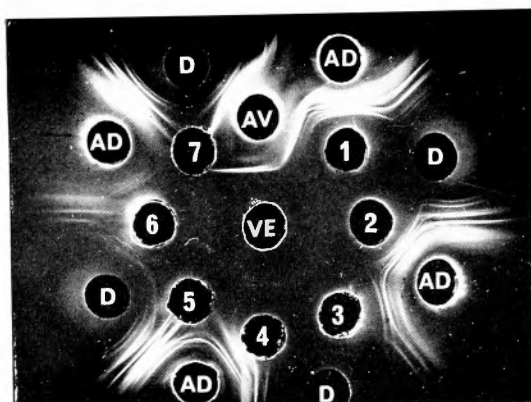
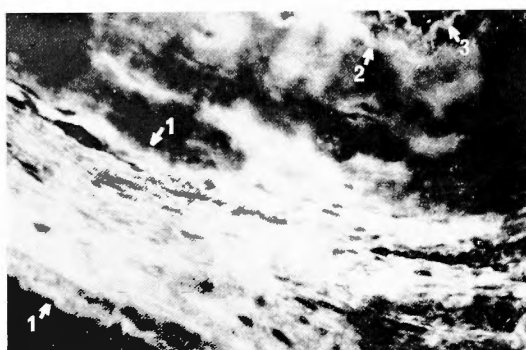


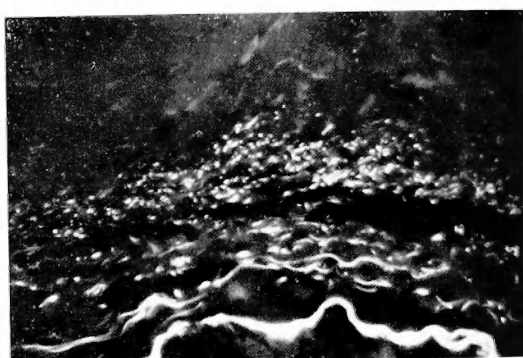
Fig. 5



Fig. 7



(1)



(2)

Fig. 6

**Fig. 5** Double gel diffusion study of emulsion of canine aortic valve, canine whole serum and their antisera. No precipitin arc is formed between the emulsion of canine aortic valve and its antiserum absorbed with successively diluted canine whole serum.

VE : emulsion of canine aortic valve

AV : anti-canine aortic valve rabbit antiserum

1, 2, 3, 4, 5, 6 : Anti-canine aortic valve rabbit antiserum absorbed with canine whole serum. The ratios of canine whole serum to anti-canine aortic valve rabbit antiserum are as follows :

1. (1:4), 2. (1:8), 3. (1:16), 4. (1:32), 5. (1:64), 6. (1:128)

7 : physiological saline

AD : anti-canine whole serum rabbit antiserum

D : canine whole serum

**Fig. 6** (1) Brightly fluorescent canine aortic valve stained with FITC-labeled anti-canine aortic valve and rhodamine-labeled anti-canine whole serum rabbit antibodies by the double staining method.

The region between the two arrows (1) is a broad area of intense fluorescence on the ventricular side of the leaflet, containing many specific fluorescent collagen fibers and some autofluorescent elastic fibers.

Arrow 2 shows a specific fluorescent thin layer of collagen fibers just beneath the endothelium of the aortic side of the leaflet.

Arrow 3 shows the brightly fluorescent endothelium on the aortic side of the leaflet.

Some faintly fluorescent scattered fibers are mixed in the nonfluorescent region, which lies between the thin layer of the fluorescent collagen fibers just beneath the endothelium of the aortic side of the leaflet and the broad intensely fluorescent part on the left ventricular side of the leaflet.

(2) Autofluorescence of elastic fibers on left ventricular side (lower) of canine aortic valve. Paraffin section stained with FITC-labeled normal rabbit gamma globulin. The collagen fibers are not stained at all with this conjugate.

**Fig. 7** Sarcolemma of canine cardiac muscle fibers intensely stained with FITC-labeled anti-canine aortic valve rabbit antibody.

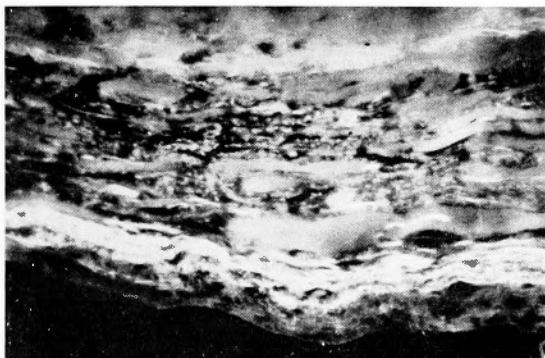


Fig. 8

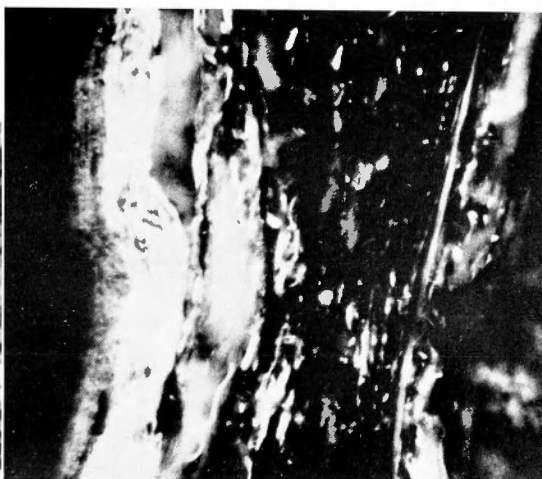


Fig. 9

**Fig. 8** Bright human serum gamma globulin-specific fluorescence of collagen fibers on left ventricular side (lower) of human aortic valve, along autofluorescence of elastic fibers.

Paraffin section stained with FITC-labeled anti-human gamma globulin rabbit antibody.  
32-year-old female.

**Fig. 9** No specific fluorescence for human serum albumin is observed in the human aortic valve. Only autofluorescence of the elastic structures is shown. The ventricular side of the leaflet is on the left.

Paraffin section stained with rhodamine-labeled anti-human serum albumin rabbit antibody.  
32-year-old female.

## PART II. EXPERIMENTAL HOMOTRANSPLANTATION OF THE AORTIC VALVE

### A. Introduction

Clinical reports<sup>3)4)5)6)7)8)9)10)11)12)</sup> have suggested that homologous aortic valves transplanted to the subcoronary position are capable of prolonged function in humans. Morphological and histological findings of such grafts were studied at various intervals after insertion by SMITH<sup>22)</sup> and HUDSON<sup>20)</sup>.

However, more detailed observations at various definite intervals after insertion should be obtained from experimental studies. Because of the technical difficulties and the high mortality rate, the information<sup>23)24)</sup> about homograft valve replacement in experimental animals is still far from adequate.

In Part II of the present studies mongrel dogs were used to demonstrate the fate of fresh homograft aortic valves after transplantation as well as the comparable fate of freeze-dried valves or those sterilized with beta-propiolactone (BPL); and to ascertain whether an immune reaction occurs against the antigenic substance demonstrated in the canine aortic valve in Part I of this study.

### B. Materials and Methods

In Series I a single cusp of the pulmonary valve and in Series II a single cusp of the aortic valve were replaced with a single cusp of the homologous aortic valve in mongrel dogs weighing 7 to 11 kilograms under surface-induced deep hypothermia.

#### 1. Preparation of the Homograft

In each series, three kinds of homograft aortic valves were transplanted: (1) fresh

valves (Group 1 and Group 4), (2) freeze-dried valves (Group 2 and Group 5), (3) BPL-treated valves (Group 3 and Group 6) (Table 1). Fresh valves were excised from the donor by sterile technique, preserved at 4 C. in 100 ml. of RINGER's solution with 50,000 units of penicillin, 100 mg. of streptomycin and 50 mg. of tetracycline, and transplanted within 6 hours after removal.

Table 1

| Method of operation                            | Method of sterilization and preservation | Dogs | Survived after operation |                |          |            |
|--|--|------|--------------------------|----------------|----------|------------|
|  |  |      | <3 days                  | 3 days ~ 5 mo. | 6~11 mo. | 12 ~24 mo. |
| Series I<br>Replacement of the pulmonary valve | Group 1 (fresh)                          | 12   | 5                        | 4              | 1        | 2          |
|  | 2 (freeze-drying)                        | 6    | 2                        | 3              | 0        | 1          |
|  | 3 (BPL)                                  | 10   | 4                        | 4              | 1        | 1          |
|  | Total                                    | 28   | 11                       | 11             | 2        | 4          |
| Series II<br>Replacement of the aortic valve   | Group 4 (fresh)                          | 9    | 3                        | 4              | 1        | 1          |
|  | 5 (freeze-drying)                        | 4    | 2                        | 1              | 1        | 0          |
|  | 6 (BPL)                                  | 7    | 2                        | 3              | 1        | 1          |
|  | Total                                    | 20   | 7                        | 8              | 3        | 2          |

Under sterile conditions, freeze-dried valves were prepared by driving a vacuum pump for about six hours after the valves were frozen in an alcohol-dryice mixture ( $-80^{\circ}\text{C}.$ ) for about 30 minutes; they were preserved in a vacuum glass tube at room temperature for four weeks. Prior to insertion, these freeze-dried valves were placed in normal saline at room temperature for approximately 30 minutes.

BPL-treated valves were sterilized with 1% BPL-solution by the method of LO GRIPPO<sup>25)</sup> and stored in 100 ml. of HANKS' solution at 4 C. for four weeks with 10,000 units of penicillin, 10 mg. of streptomycin and 5 mg. of tetracycline.

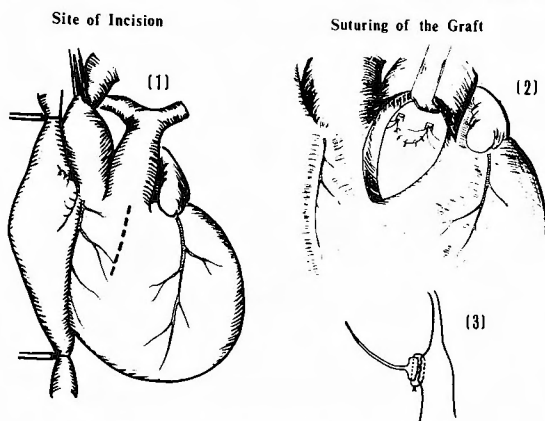
As much of the cardiac muscle and aortic wall adjacent to the valve was excised as possible for the purpose of obtaining complete cooperation with the other two host valves so as not to cause any stenosis or incompetence after insertion. All donor dogs weighed a little less than the recipients.

## 2. Operative Techniques

For seven to ten days preoperatively, the animals were fed Soya-Lecithin (0.5 g/kg/day) as a source of essential fatty acids<sup>26)27)</sup>. After premedication with 0.2 mg. of atropine, intubation was followed by the intravenous injection of 2 to 3 mg. per kilogram of thiopental sodium and 0.4 mg. per kilogram of succinylcholine chloride. The recipient was cooled in ice water to 29 C. (average rectal temperature) for pulmonary valve replacement and to 24 C. for aortic valve replacement. During the cooling process, shivering was prevented by careful deep OE (oxygen and ether) anesthesia. The after-drop of the rectal temperature was usually 3 to 6°C. Therefore, the circulation was arrested at 25.2°C. (average rectal temperature) in the first series and at 19.4 C. in the second series.

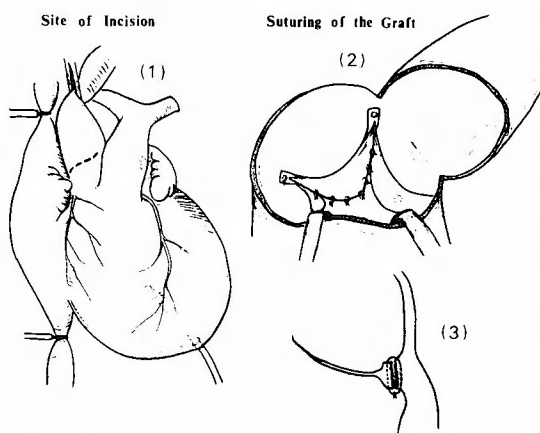
Following occlusion of the superior and inferior venae cavae and the aorta without heparinization, complete cardiac arrest was obtained by the rapid injection of 1 ml/kg. of

YOUNG's solution ( $0.54 \text{ g. K}_3(\text{C}_6\text{H}_5\text{O}_7) \cdot \text{H}_2\text{O}$ , potassium citrate,  $2.47 \text{ g. MgSO}_4 \cdot 7\text{H}_2\text{O}$ , magnesium sulfate in  $100 \text{ ml.}$  of distilled water,  $\text{pH } 7.4$ ) at  $4.0^\circ\text{C.}$  into the aortic root. In the first series, the posterior semilunar cusp of the pulmonary valve was replaced and in the second series, the non-coronary cusp of the aortic valve was replaced with a homologous aortic single-cusp (Fig. 10, 11). In each series, the graft was secured with interrupted nylon sutures. During replacement, the right ventricle in Series I and the left ventricle in Series II were cleared by heparinized normal saline which was washed out by non-heparinized normal saline, just before the closure of the right ventricle or the aorta.



**Fig. 10** Operative techniques of replacement of the pulmonary posterior semilunar cusp with homologous aortic valve.

- (1) Dotted line shows the site of incision on the out-flow tract of the right ventricle. (2) Appearance after the completion of the transplantation. (3) Technique of securing the graft.



**Fig. 11** Operative techniques of replacement of the aortic non-coronary cusp with homologous aortic valve.

- (1) Dotted line shows the site of incision in the aortic root. (2) Appearance after the completion of the transplantation. (3) Technique of securing the graft.

Immediately after the completion of replacement and closure of the right ventricle or the aorta, cardiac massage was performed for resuscitation under the intrathoracic and surface rewarming in a warm water bath. Electric counter shock was used for defibrillation, if necessary. Pure  $\text{O}_2$  was inhaled during the rewarming phase. When the rectal temperature reached  $30^\circ\text{C.}$ , intrathoracic rewarming was stopped and the chest was closed. After that, surface rewarming was continued until the rectal temperature was  $36^\circ\text{C.}$  Figs. 12 and 13 show the typical record of hypothermia in each series.

Postoperatively, no immunosuppressive drugs or anticoagulants were used in all animals.

### 3. Examination after Operation

Angiographic and cardiac catheterization studies were performed on selected dogs in each series and group at varying intervals after operation in order to assess the function of the transplanted valve.

Complete autopsy studies were done in all of the animals that died or were sacrificed. All valves were stained with hematoxylin and eosin, elasticVAN GIESON, phosphotungstic acid hematoxylin (PTAH) and other stains as needed, and examined by light microscopy.

Some sections were studied under a Carl Zeiss fluorescence microscope after the fixation and staining procedures described in Part I.

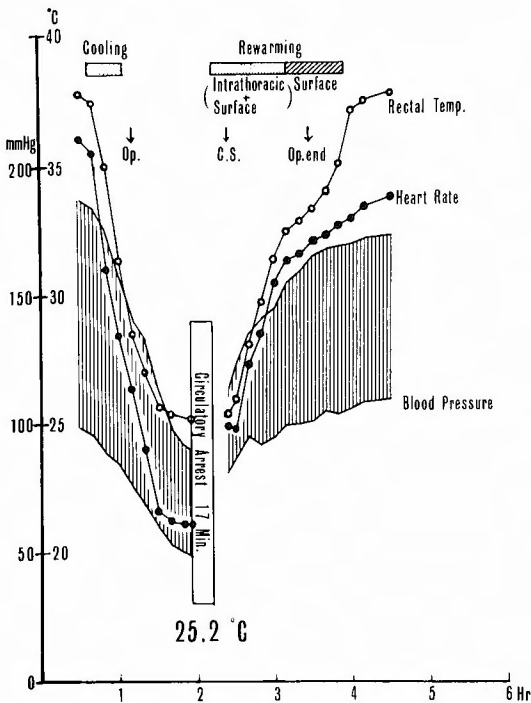


Fig. 12 Typical record of hypothermia for replacement of a single cusp of the pulmonary valve with homologous aortic valve (Series I).

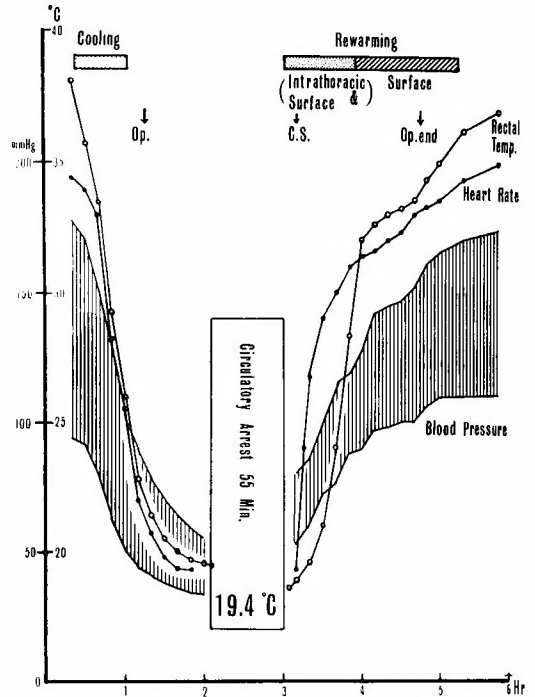


Fig. 13 Typical record of hypothermia for replacement of a single cusp of the aortic valve with homologous aortic valve (Series II).

### C. Results

#### 1. Operative Results

The operative mortality was much higher in the dogs receiving aortic valve replacement (Series II) than in those with a pulmonary valve replacement (Series I). In Series I, 28 of 40 dogs survived or were sacrificed between 24 hours and 24 months after operation, while in Series II, 20 of 52 dogs survived or were sacrificed (Table 1).

The most frequent cause of death in both series was the presence of *Filaria* in the venous system which interfered with successful resuscitation after the completion of the replacement. The higher operative death in Series II than in Series I was due not only to the frequent occurrence of cardiac arrest or ventricular fibrillation during cooling, but also to bleeding from the suture line of the aorta during rewarming.

#### 2. Gross Morphologic Features of the Grafts, and Catheterization and Angiographic Findings

##### (1) Pulmonary valve single-cusp replacement (Series I)

In all three groups, all of the valves had a very similar appearance at all examinations between three and 24 months after insertion.

Up to 30 days after operation, the leaflets still remained somewhat pliable with moderate thickening, but at three months they had almost lost their pliability with severe thickening, and at six months had lost it completely with severe scarring, retraction and calcification.

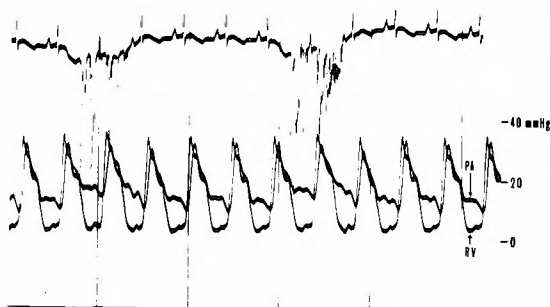


**Fig. 14** Macroscopic findings of a fresh homologous aortic single cusp (arrow) examined 24 months after transplantation to the pulmonary valve area. The graft retains its pocket-shape, but is thickened and immobile.

## (2) Aortic valve single-cusp replacement (Series II)

In comparison with Series I, all transplanted valves in Series II retained their pliability, and functioned perfectly as valves up to 24 months after transplantation in Groups 4 and 6 (Fig. 17), and up to six months in Group 5.

The two fresh grafts (Group 4), examined at 25 and 65 days postoperatively showed slight thickening and edema and had lost some of their semitranslucency. However, an almost normal appearance and pliability, except for thickening at the suture line, was observed in fresh valves examined six and 24 months after operation.



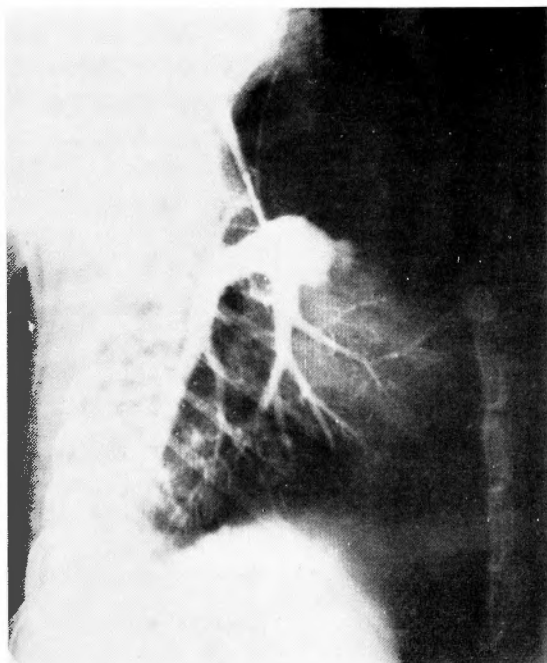
Operation ; Oct. 9 '65  
Record & Sacrifice ; Oct. 25 '67

**Fig. 15** Simultaneous pressure recording from right ventricle and pulmonary artery 24 months after a fresh homologous aortic single cusp had been transplanted to the pulmonary valve area. Neither stenosis nor incompetence of the pulmonary valve is significantly revealed.

In spite of such evidence of severe degeneration, these grafts kept their pocket-shape as cusps, and remained unrejected even 24 months after transplantation (Fig. 14).

Catheterization and angiographic studies after six and 24 months in Groups 1 and 3, and after 24 months in Group 2 revealed neither significant stenosis nor incompetence of the pulmonary valve (Fig. 15, 16).

Fibrin deposition and thrombi were occasionally demonstrated in early postoperative specimens, but no gross thrombi were observed in specimens examined more than three months after insertion.



**Fig. 16** Angiogram (lateral projection) of the main pulmonary artery in the same animal as Fig. 15, 24 months after insertion. No incompetence of the pulmonary valve is revealed.

On the other hand, BPL-treated valves (Group 6) showed no thickening or edema even in the early postoperative period (three days to five months) and retained better pliability than fresh grafts, but at later postoperative periods (six to 24 months) there was no marked difference in the gross morphologic features of fresh and preserved valves.

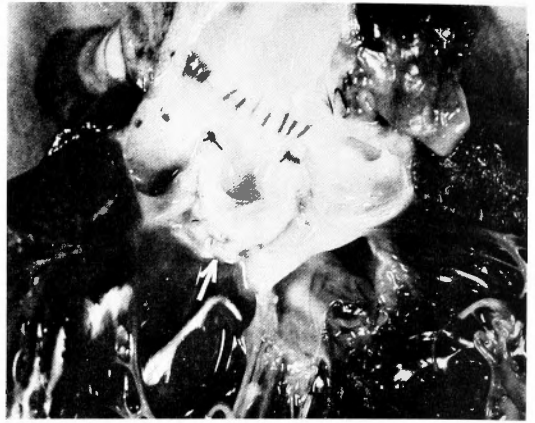
Small thrombus formations were observed at the suture line in the early postoperative specimens in all groups of Series II, but after three months, no gross thrombi were demonstrated in any of the three groups.

In the animals which survived for more than three days, large or small vegetations formed on the infected leaflet in most of the specimens of Group 4 (fresh) and 5 (freeze-dried), but in only one of Group 6 (BPL-treated).

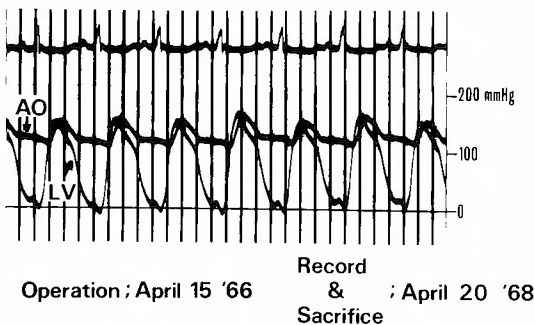
Aortic angiography and arterial catheterization were performed in two cases (at six and 24 months) of Group 4, in one case (at six months) of Group 5, and in two cases (10 and 24 months) of Group 6 (Fig. 18, 19). These studies showed no aortic incompetence in any case, but slight aortic stenosis was noted in one case (24 months) of Group 4, and in one case (six months) of Group 5, and in these two cases the transplanted valves were partially infected.

### 3. Histologic Features

Bacterial infection was evident in many of the grafts, especially in Groups



**Fig. 17** Macroscopic findings of a BPL-treated homologous aortic single cusp (arrow) examined 24 months after transplantation to the subcoronary position. The transplanted valve is thin and pliable.



**Fig. 18** Simultaneous pressure recordings from left ventricle and ascending aorta 24 months after a BPL-treated homologous aortic single cusp had been transplanted to the subcoronary position. Neither stenosis nor incompetence is revealed.



**Fig. 19** Angiogram (lateral projection) of the ascending aorta in the same animal as Fig. 18, 24 months after insertion. No incompetence of the aortic valve was revealed.



4 and 5. However, the following descriptions refer to the apparently non-infected areas of the grafts.

(1) Pulmonary valve single-cusp replacement (Series I)

(a) Fresh graft (Group 1)

The valves examined up to four weeks after insertion were moderately thickened and edematous with proliferation and hyalinization of collagen fibers, and separation of the elastic lamellae. Severe infiltration of polymorphonuclear leucocytes and lymphocytes was found at the graft-host junctional zone as well as slight, scattered infiltrations of these cells in the leaflets, but this cell infiltration had almost disappeared three months postoperatively. After four weeks, the collagen fibers tended to hyalinize, and in these degenerated collagen fibers the formation of cartilaginous tissues could be seen. These degenerations of collagen fibers were observed at the base of the leaflet at first and then at the apex, as time passed after the operation. The leaflets examined six months postoperatively were occupied mostly by cartilaginous tissue in the middle of the leaflet as well as at the base of the valve (Fig. 20). Granulation tissues with abundant blood vessels extended on both surfaces for half the length of the valve six months postoperatively. At 15 and 24 months after insertion, even at the apex of the cusps, the collagen fibers showed cartilaginous formation, and the central part of these tissues contained many calcium deposits. These cartilaginous tissue were surround by a thin layer of granulation tissue covered by endothelial cells (Fig. 21). Elastic lamellae were severely separated, remaining around the cartilaginous tissues, and seemed to be less numerous than in grafts examined earlier (up to three months postoperatively). Mild infiltration of plasma cells, lymphocytes and histiocytes was demonstrated along the graft-host junction in the specimens up to three months, but even at these early postoperative periods plasma cells or large mononuclear cells were never seen on the leaflets. Occasionally foreign body giant cells in the graft-host junctional zone were seen close to suture materials.

(b) Freeze-dried and BPL-treated grafts (Groups 2 and 3)

The histologic findings of freeze-dried and BPL-treated valves were very similar. All the nuclei of the donor cells, including endothelial cells had disappeared by one to two weeks after transplantation with severe degeneration of the valve tissue. The extension of granulation tissue to these preserved valves was slower than to the fresh valves. Six months postoperatively the valves remained acellular except in the area of granulation near the base of the cusp. In the donor's collagen fibers there was formation of fibrocartilaginous or caltilaginous tissues, similar to that in the fresh valves, but in the preserved valves, especially in the BPL-treated valves, the degeneration of collagen fibers was less pronounced than in the fresh valves, even 24 months after operation (Fig. 22). The granulation tissues also surrounded most of the surface of the valve 24 months postoperatively.

The infiltration of neutrophilic leucocytes and other inflammatory cells was less severe than in the fresh valves, even at the graft-host junctional zone in the early postoperative stage.

(2) Aortic valve single-cusp replacement (Series II)

(a) Fresh valve (Group 4)

Leaflets of fresh grafts demonstrated slight edematous thickening up to four months

after operation, but thereafter there was no further thickening and even some decrease in thickness.

Most of the nuclei in the valve became pale at ten days and almost disappeared at 65 days except for a few pyknotic nuclei near the base of the cusp.

The endothelial lining had already disappeared at 25 days.

Signs of degeneration of collagen fibers, such as cloudy swelling and gradual disappearance of the nuclei of the cells, were observed as early as 10 days after transplantation. The valves examined after four, six and 24 months were composed only of these degenerated collagen fibers except for the areas replaced by host tissues (Fig. 23).

In the process of replacement of the graft by host tissues, early regeneration of the endothelium was seen only near the base of the cusp examined four months postoperatively, but this endothelial lining had extended over half of the leaflet from the base at six months. At 24 months after insertion, the regenerated endothelium completely covered both surfaces of the valve (Fig. 23). At four months, the leaflet was still acellular, but by six months after operation fibroblastic tissues were seen replacing one third of the valve from the base, chiefly on the ventricular side of the leaflet. By 24 months, these tissues had extended to the area near the apex of the valve (Fig. 24). In these areas of replacement of the leaflet, no blood vessels were seen except close to the base of the valve.

Only at the graft-host junctional zone, the collagen fibers of the donor valve showed marked cartilaginous metaplasia more than one month postoperatively, but this was limited to the base of the valve, and did not extend into the leaflet even 24 months postoperatively.

Elastic lamellae were loosely composed and separated, but remained almost intact without fragmentation up to 24 months after operation.

There was marked infiltration of neutrophilic leucocytes and other inflammatory cells in the graft-host junctional area and minimal infiltration in the leaflets up to one month after operation. Later (after two, four and six months), a few plasma cells and lymphocytes were seen in the granulation tissues at the junctional zone, but no round cell infiltration was observed in the leaflet.

For up to two months focal thrombi were seen on the surface of the valve, but these were never seen later, and focal thin layers of fibrin without calcification were demonstrated in the leaflet only microscopically by PTAH stain.

#### (b) Freeze-dried and BPL-treated valves (Groups 5 and 6)

The nuclei of the cells in normal aortic valves after treatment of the valve by freeze-drying or immersion in 1% BPL-solution, already appeared more pyknotic than those in fresh, non-treated aortic valves. By six days after insertion, both kinds of preserved valves were virtually acellular and remained bare, having lost its endothelial cells. Thus, the nuclei in the preserved valves disappeared earlier than those in the fresh valves.

Later degeneration of collagen fibers and separation of elastic lamellae (at six months in Group 5, and five, 10 and 24 months in Group 6) resembled those of fresh grafts examined at corresponding postoperative periods. Even after 24 months these preserved homograft aortic valves remained pliable without thickening or calcium deposition in the leaflet (Fig. 25). Only at the graft-host junctional zone, marked cartilaginous or fibro-

cartilaginous metaplasia of the collagen fibers in the donor valve was observed five months postoperatively, as when fresh homograft aortic valves were used.

A small degree of regeneration of host endothelium was observed at the base of the BPL-treated valve at five months and of the freeze-dried valve at six months. Fibroblastic tissues were also seen at the base of these two valves. Ten months after insertion, the host endothelium covered the entire surface of the BPL-treated valve (Fig. 26), and fibroblastic tissues extended to half the valve from the base (Fig. 27). Furthermore, by 24 months after insertion, the leaflets were replaced from the base to the apex by these fibroblastic tissues, chiefly along the elastic fibers of the ventricular side of the leaflet. In these fibroblastic tissues, including the regenerated endothelial cells, acid mucopolysaccharide was demonstrated by alcian blue stain on all the leaflets examined, but not in the acellular or non-replaced areas (Fig. 28).

No plasma cells or large mononuclear cells were ever seen in any of the leaflets examined, but there was a slight infiltration of polymorphonuclear leucocytes up to one week after operation.

#### 4. Studies by Fluorescent Antibody Technique

Normal BPL-treated canine aortic valves showed no fluorescence when stained with FITC-labeled anti-canine aortic valve rabbit antibody, which was the same conjugate as that used in Part I of this study. Therefore, this test could demonstrate the degree of replacement of the graft by host tissues; and valves which had been treated with BPL before transplantation were stained with anti-canine aortic valve conjugate.

The BPL-treated valves examined 10 and 24 months after operation showed bright fluorescence at the same location in which fibroblastic tissues were observed under the light microscope (Fig. 29). On the other hand, except in the endothelial cells, there was no specific fluorescence near the apex of the same valve, where no fibroblastic tissue was seen and where the valve remained acellular except for the endothelial cells (Fig. 30). These findings definitely confirmed that these fibroblastic tissues, observed under light microscopy, were derived from the host tissues.

Heterotopically transplanted valves (to the pulmonary valve area), sterilized with 1% BPL-solution before operation, were also examined by the same technique. Strong fluorescence was seen in all parts of the valve tissues (24 months), except in the cartilaginous tissues at the central part of the valve (Fig. 31). Therefore, it was also apparent that most of the donor valve tissues, except for the cartilaginous tissues, were replaced by host granulation tissue.

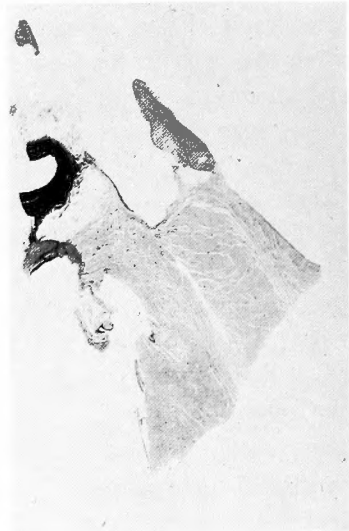
#### *D. Discussion*

Because of the high operative mortality and high incidence of infection in homografts, little of the hoped-for information has been obtained concerning the histological fate of aortic valve homografts.

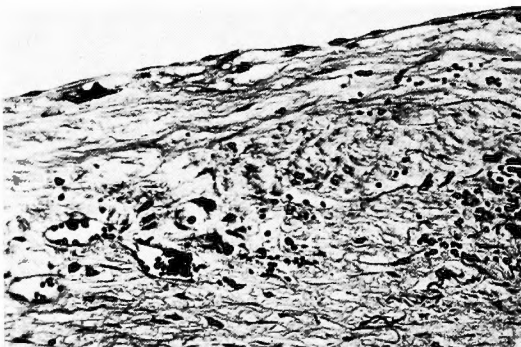
However, the present study, using fluorescent antibody techniques as well as the usual histological examination, clearly demonstrated the durability of homograft aortic valves without loss of function or immunological problems for up to two years and the replacement of the graft by host tissues. No previous report has described the replacement of the transplanted valve by host tissues.



**Fig. 20** Fresh aortic valve homograft in pulmonary valve area, six months after operation; photomicrograph of valve homograft. The middle part as well as the base of the markedly thickened cusp is occupied by cartilaginous tissue. Markedly separated elastic lamellae are also seen on the ventricular side (lower left) of the cusp (elastic VAN GIESON stain;  $\times 1$ ).



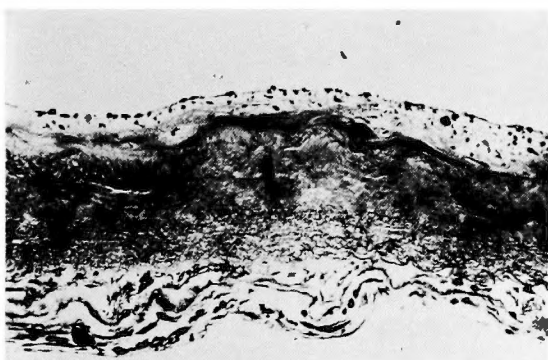
**Fig. 22** BPL-treated aortic valve homograft in the pulmonary valve area 24 months after operation; photomicrograph of the valve homograft. Although fibrocartilaginous formations are seen in the valve, thickening of the cusp is not so severe as that of fresh valves examined six months after operation (Fig. 20) (elastic VAN GIESON stain;  $\times 1$ ).



**Fig. 21** Granulation tissue and endothelial covering on the pulmonary side of a fresh aortic valve homograft in the pulmonary valve area, 24 months after operation (hematoxylin and eosin stain;  $\times 200$ ).



**Fig. 23** Endothelial cells at the apex of a fresh aortic valve homograft examined 24 months after orthotopic transplantation (hematoxylin and eosin stain;  $\times 400$ ).



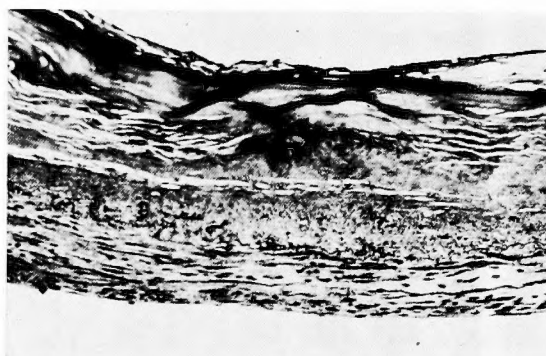
**Fig. 24** Fibroblastic tissues near the apex of a fresh aortic valve homograft 24 months after orthotopic transplantation (hematoxylin and eosin stain ;  $\times 100$ ).



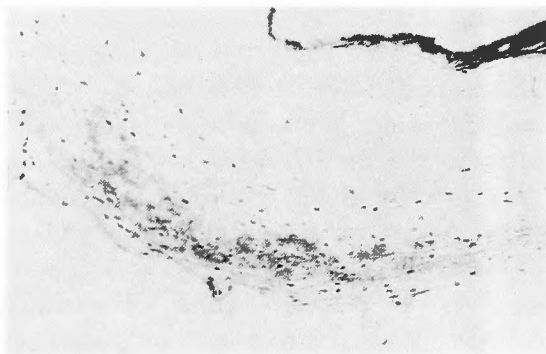
**Fig. 25** BPL-treated aortic valve homograft in the subcoronary position 24 months after operation ; photomicrograph of valve homograft. The leaflet is thin and pliable without calcium deposition. Cartilaginous tissues are seen at the base of the valve (elastic VAN GIESON stain :  $\times 1$ ).



**Fig. 26** Endothelial cells at the apex of a BPL-treated valve 10 months after orthotopic transplantation. Collagen fibers in the valve show cloudy swelling and are acellular (hematoxylin and eosin stain ;  $\times 400$ ).



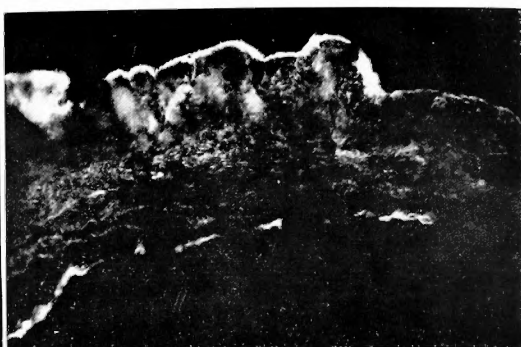
**Fig. 27** Fibroblastic tissues chiefly on the ventricular side (lower) of a BPL-treated aortic valve homograft 10 months after orthotopic transplantation (hematoxylin and eosin stain :  $\times 100$ ).



**Fig. 28** Acid mucopolysaccharide is shown on the aortic (upper) and ventricular side (lower) of a BPL-treated aortic valve homograft 10 months after orthotopic transplantation (alcian-blue stain ;  $\times 100$ ).



**Fig. 29** Bright fluorescence is shown by staining with FITC-labeled anti-canine aortic valve rabbit antibody at the place where fibroblastic tissues were observed under light microscopy in a BPL-treated aortic valve homograft 10 months after orthotopic transplantation. Ventricular side of the valve is left.



**Fig. 30** The apex of a BPL-treated aortic valve homograft (10 months after orthotopic transplantation), which was acellular except for regenerated endothelium, is not stained at all with FITC-labeled anti-canine aortic valve rabbit antibody except for the endothelial cells. Ventricular side of the valve is lower.

This study also showed that when aortic single-cusp homografts were transplanted to the pulmonary valve area, both viable and nonviable valves became greatly thickened and lost their ability to function as valves. These findings agree with those of LOWER et al.<sup>24)</sup> The reason is not apparent why these valves lost their pliability in contrast to the valves transplanted to the subcoronary position, but it is true that a homograft aortic valve transplanted to the pulmonary valve area is less forced to work by blood pressure than a valve transplanted to the subcoronary position. Moreover, even when this heterotopically transplanted valve is immobile, the right ventricle is able to tolerate slight pulmonary



**Fig. 31** Granulation tissue in a BPL-treated homograft aortic valve transplanted to the pulmonary valve area (24 months) is stained intensely with FITC-labeled anti-canine aortic valve rabbit antibody, but cartilaginous tissue (left) is not stained at all.

stenosis, and the recipient's own two remaining pulmonary cusps easily compensate for the poor function of the transplanted valve and prevent pulmonary regurgitation. Hence, an aortic single-cusp transplanted to the pulmonary valve area seems to become immobile and thickened. This conjecture is also supported by the experiments of LAM and associates<sup>25)</sup>, in which the homograft aortic valve transplanted to the descending thoracic aorta lost its function unless the recipient's own aortic valve was incompetent. Therefore, it may be that if all three pulmonary semilunar cusps are replaced by homologous aortic cusps they will retain pliability and continue to function for long periods.

LOWER et al.<sup>24)</sup> reported the prolonged existence of the nuclei of the fibrocytes in fresh homograft aortic valves after orthotopic transplantation. However, in this study the



nuclei of the cells in fresh valves disappeared as early as two months after insertion. It seems that these differences depend on the physical and chemical injury to the valves during the preservation and operative procedures. In clinical cases, SMITH<sup>22)</sup> reported that a fresh valve stored for three days in nutrient medium was cellular nine months after insertion, but four other valves stored for between 11 and 24 days were acellular over three months after operation. MOHRI et al.<sup>23)</sup> reported in their experimental study that cellular and acellular portions were seen in one section examined 10 months postoperatively. They attributed this finding to mechanical injury at the time of graft insertion. It is considered that the method of preservation of fresh valves used in the present study was so toxic to valve tissues that the disappearance of the nuclei in the valve occurred early after operation.

Regeneration of true endothelial cells was not seen in fresh, freeze-dried or BPL treated valves in clinical cases up to two years after operation by LOWER<sup>24)</sup> and SMITH<sup>22)</sup>. However, in the present study regeneration of true endothelium was noted at the base of BPL-treated valves at five months and on all surfaces of this kind of valve at 10 months after insertion. MOHRI et al.<sup>23)</sup> also observed a small area of extension of host endothelium on to the base of the leaflet of BPL-treated valves at three months. It seems, therefore, that there are some differences in tissue regenerative reactivity between humans and dogs. This difference is also apparent in the replacement of the transplanted valve by host fibroblastic tissues. None of the clinical reports<sup>20) 22)</sup> has demonstrated so early an appearance and so long an extension of host fibroblastic tissues as the BPL-treated valves after five and 10 months and freeze-dried valve after six months in the present experiment.

In regard to the immunological factors, there was a significant infiltration of plasma cells, lymphocytes and large mononuclear cells at the host-graft junctional zone of fresh valves and only minimal infiltration at the same zone of the preserved valves in the early postoperative periods. The fibrous tissues at the base of normal canine aortic valves contain antigenic substances as described in Part I, so that it may be possible to attribute these infiltrates to the immune reactions. However, the other antigenic collagen fibers of the valve, which are chiefly among the elastic fibers on the ventricular side of the leaflet, play almost no part in immune reactions after the insertion of fresh grafts.

There are several reasons why the collagen fibers of donor valves transplanted to the subcoronary position showed cartilaginous metaplasia at the graft-host junctional zone, even with preserved valves and even though the leaflet retained sufficient pliability. The two major causes are probably immobility of the base of the valve and rapid enclosure of the donor valve tissues at the valve base by granulation tissues containing abundant blood vessels.

These various facts suggest that the pliability and functional ability of orthotopically transplanted homograft aortic valves for long periods depend on the relative avascularity of the leaflet, the low antigenicity of cardiac valves and enforced movement of the transplanted valve by high blood pressure.

#### *E. Summary*

1. In one experiment single cusps of canine pulmonary valves and in another single cusps of aortic valves were replaced by homologous aortic valve cusps.

2. In each series, three kinds of homologous aortic valve were transplanted: fresh,



freeze-dried and BPL-treated valves.

3. All of the homologous aortic valves transplanted to the pulmonary valve area became very much thickened and retracted with calcium deposits, and had lost their valve function six months postoperatively. These transplanted valves, however, retained their pocket-shape up to 24 months after insertion without rejection.

4. The homologous aortic valves transplanted to the subcoronary position remained pliable with only slight thickening and kept on functioning for up to six months (freeze-dried valve) or 24 months (fresh and BPL-treated valves) after insertion, even though the graft was replaced by host tissues.

5. There were some differences in the morphologic and histologic findings between the fresh and the preserved valves after insertion: (a) When the valves were transplanted heterotopically, degeneration of the collagen fibers of the valve was more severe in the fresh than in the preserved valves. (b) The nuclei of the cells in the preserved valves disappeared earlier in the postoperative period than those of the fresh valves. (c) By 4 months after operation, the orthotopically transplanted fresh valves were slightly edematous, in comparison with the preserved valves. (d) At the graft-host junctional zone, the infiltration of plasma cells, lymphocytes and large mononuclear cells was greater in the fresh than in the preserved valves.

6. Regeneration of true endothelium on the leaflets and replacement of the valves with host fibroblastic tissues occurred to some degree in all specimens beyond six months after orthotopic homotransplantation of the aortic valve.

7. In the heterotopically transplanted valves, abundant blood vessels were seen in the replaced area, but this occurred in the orthotopically transplanted valves only close to the base of the valve.

8. Marked separation of the elastic lamellae was noted in the heterotopically transplanted valves, but only moderate separation in the orthotopically transplanted valve. In all cases, however, the elastic fibers remained unfragmented up to 24 months after insertion.

9. Even when the grafts were fresh, no significant immune reaction was observed in the leaflets of either heterotopically or orthotopically transplanted valves, except at the graft-host junctional zone.

### **PART III. CLINICAL EXPERIENCE WITH HOMOGRAFT AORTIC VALVE REPLACEMENT**

#### *A. Introduction*

Thorough fundamental experiments, described in Part I and Part II, have demonstrated that in spite of the existence of some antigenic substances in canine aortic valves, canine homograft aortic valves transplanted to the subcoronary position continued to function without loss of pliability or significant immune reaction, whether the graft was viable or nonviable.

On the basis of these experimental results, four clinical homograft aortic valve replacements have been performed in our clinic, the Second Surgical Division, Department of Surgery, Kyoto University Medical School, since October, 1967.

#### *B. Preparation of Homografts*

The homograft aortic valves, including some adjacent tissues such as aortic wall and

myocardium, were obtained from cadavers under non-sterile conditions within 6 hours after death and stored at 4°C. until sterilization was performed within 12 hours after excision. Donor valves were not taken if the patients had had severe jaundice, a positive WASSERMAN test or septicemia. Moreover, valves were discarded if they were deformed, stenotic, incompetent, or had atheromas either on the leaflet or at the base of the cusp. Therefore, acceptable homografts were obtained only from donors under 40 years of age.

Valves were sterilized at 37°C. for 3 hours in 1% beta-propiolactone (BPL)-solution, according to the method of LO GRIPPO<sup>25)</sup>. After sterilization, the homograft was washed three times with cold physiological saline under sterile conditions and stored at 4°C. in 250 ml. of HANKS' solution, containing 20,000 units of penicillin, 20 mg. of streptomycin and 10 mg. of tetracycline. Samples for bacterial culture were taken from (1) the beta-propiolactone solution, (2) the saline washings, and (3) the valve itself (bits of muscle and aortic wall) after washing; these samples were incubated in thioglycolate broth for two weeks at 37°C. The homografts were discarded if not used within six weeks after sterilization.

### C. Operative Techniques

The chest was opened by a median sternotomy and if some operative procedures were necessary for the associated mitral valve disease, a right antero-lateral incision in the fourth intercostal space was added.

Under extracorporeal circulation, patients were cooled to approximately 30°C (rectal temperature).

BARRATT-BOYES' transplantation technique<sup>10)13)</sup>, including vertical mattress suturing, was used mainly in this study (Fig. 32, 33). When the diameter of the recipient annulus of the aortic valve exceeded that of the available donor valves, the technique of aortic root tailoring was also employed. However, in the present study, instead of continuous sutures, interrupted mattress sutures were used for securing the lower line of the graft, about 4 mm beneath the lowest point of the cusp remnant (Fig. 32 (1, 2)).

The graft was trimmed by means of the technique of BARRATT-BOYES, except that the circular lower ring of graft tissue was left about 7mm. below the lowest point

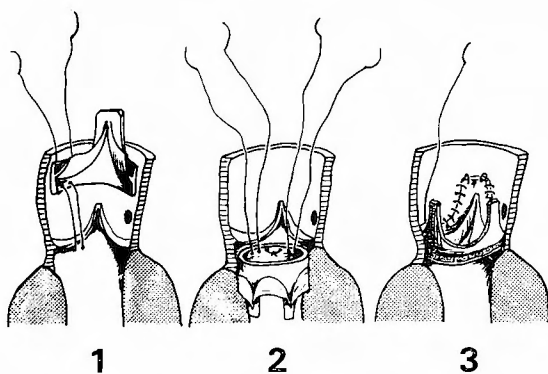


Fig. 32 Operative technique of insertion of homograft aortic valve in the subcoronary position.

1, 2 : Securing of lower line of the graft with interrupted mattress sutures.

3 : Securing of upper line of the graft with continuous sutures

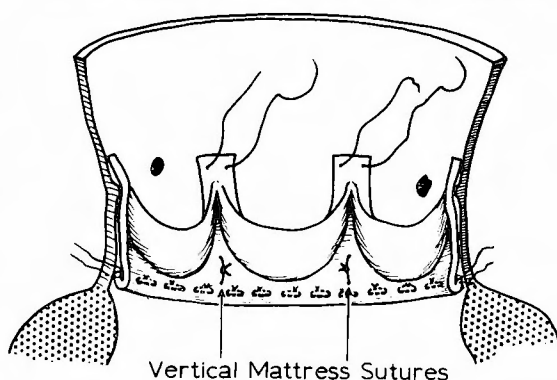


Fig. 33 Vertical mattress sutures below each of the three commissures.

of cusp attachment, a little longer than in his method.

Average bypass time was three hours and 42 minutes, and the average coronary perfusion time was two hours and 46 minutes (Table 2).

Postoperatively, no immunosuppressive drugs or anticoagulants were employed in all patients.

**Table 2** Summary of Patients Studied

| Patient | Age | Sex | Date of operation | Diagnosis      | NYHA class | Bypass time | Coronary perfusion time | Result                      | Remark                                    |
|---------|-----|-----|-------------------|----------------|------------|-------------|-------------------------|-----------------------------|---|
| 1       | 17  | M   | Oct. '67          | AI             | III        | 4° 8'       | 2° 50'                  | Died on the 3rd postop. day | Acute renal insuff. & right vent. failure |
| 2       | 24  | M   | March '68         | AI + (MI)      | III        | 3° 40'      | 3° 10'                  | Excellent                   | No treatment for MI                       |
| 3       | 29  | M   | April '68         | AS + (AI) + MS | III        | 3° 40'      | 2° 25'                  | Excellent                   | Commissurotomy for MS                     |
| 4       | 22  | M   | May '68           | AI + (AS)      | IV         | 3° 20'      | 2° 40'                  | Good                        | Tailoring., Postop.—mild AI               |

#### D. Patients (Table 2)

Between October 1967 and June 1968 four patients were treated by excision of the diseased aortic valve and transplantation of a homograft aortic valve. All patients were males aged 17 to 29 years. Two patients had predominant aortic incompetence, one had predominant aortic stenosis and one had isolated aortic incompetence. One patient required commissurotomy for associated mitral stenosis. There was a history of rheumatic fever in two patients and of bacterial endocarditis in one. All patients belonged to class III or class IV (NYHA).

#### E. Results (Table 2)

The first case, a 17-year old male, who had severe aortic valve incompetence, expired on the third postoperative day, but the other three cases, had uneventful postoperative courses and were discharged in excellent condition.

In the first case, poor right coronary perfusion during transplantation and prolonged extracorporeal circulation caused right ventricular failure and acute renal insufficiency after operation in spite of great improvement in his hemodynamics immediately after operation (Table 3). Autopsy study demonstrated that the transplantation technique was excellent,

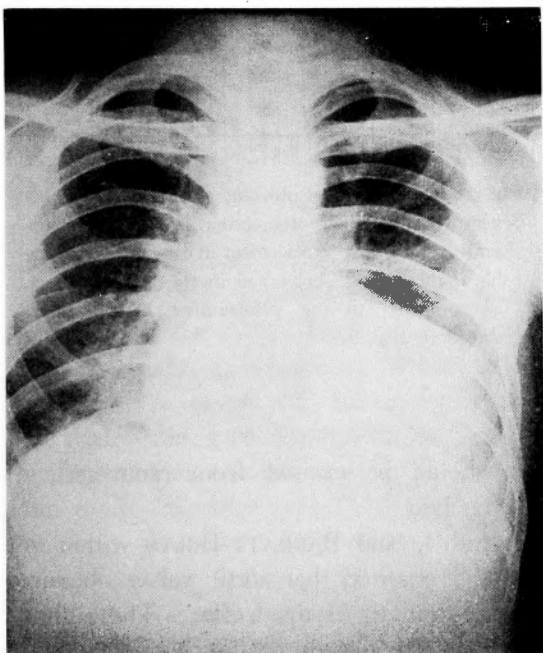
**Table 3** Hemodynamic Data Before, and Immediately After Operation

| Patient | Diagnosis      |         | Aortic blood pressure | Syst. pressure gradient between left vent. and aorta |
|---------|----------------|---------|-----------------------|--|
| 1       | AI             | Preop.  | 106/14                | 9  |
|         |                | Postop. | 150/95                | 0  |
| 2       | AI + (MI)      | Preop.  | 142/16                | 0  |
|         |                | Postop. | 126/68                | 0  |
| 3       | AS + (AI) + MS | Preop.  | 95/45                 | 55   |
|         |                | Postop. | 125/90                | 7  |
| 4       | AI + (AS)      | Preop.  | 97/25                 | 24   |
|         |                | Postop. | 115/76                | 0  |

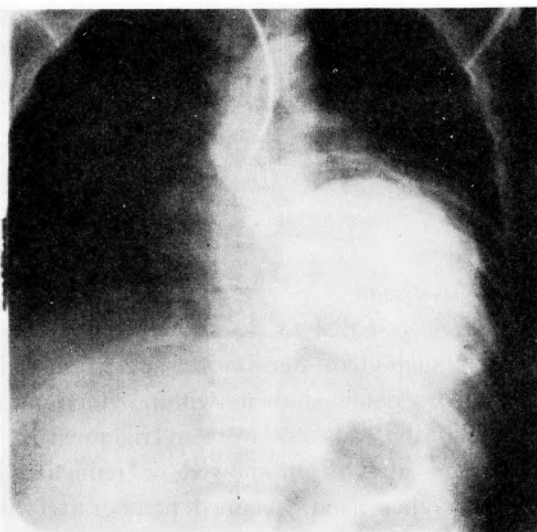
and that the cusps were slightly thickened and had lost their semitranslucency, but they were pliable, and there were no thrombi on the leaflet. Microscopic examination of the graft revealed that there was mild infiltration of polymorphonuclear leucocytes and lymphocytes at the host-graft junctional zone, and on the leaflet there were many red cells and very few lymphocytes and polymorphonuclear leucocytes, but no plasma cells or large mononuclear cells. In the leaflet all nuclei of the fibroblasts as well as endothelial cells had already disappeared and the graft remained completely acellular.

The other three cases also showed remarkable improvement in their hemodynamics immediately after operation (Table 3). In case 2, with AI and MI, the mitral valve was completely examined under direct vision during the operation. No morphological abnormality of the leaflet was seen, except for slight dilatation of the annular ring, so the mitral valve was left without any operative treatment. The systolic murmur due to mitral valve insufficiency heard before operation, had disappeared completely by one month after operation.

In case 4, the patient had already suffered from left ventricular decompensation for two weeks before operation, and his chest X-ray film (Fig. 34) and electrocardiogram showed extreme left ventricular hypertrophy. Aortography also showed severe regurgitation into the left ventricle (Fig. 35). At operation, his aortic valve was found to be bicuspid and severely affected by the previous bacterial endocarditis, with thickening, scarring and retraction, and with vegetation, calcium deposition and a perforation 5 mm. in diameter in one cusp. In this case, the severely dilated aortic root needed tailoring, and the internal diameter of 30mm. was reduced to 26 mm. In spite of his serious preoperative condition, the postoperative course was uneventful and hemodynamics was



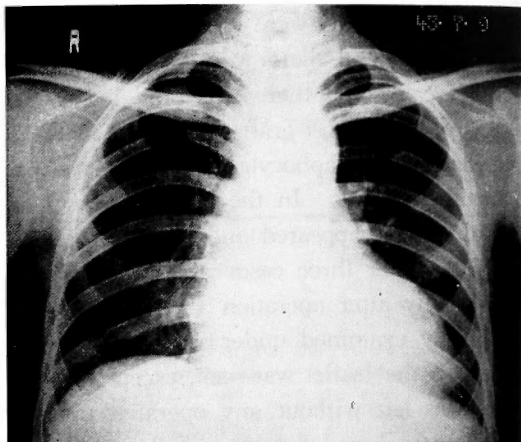
**Fig. 34** Preoperative chest X-ray film (postero-anterior projection) of case 4. Severe dilatation of the heart and increased pulmonary vasculature are observed.



**Fig. 35** Aortography of case 4 (antero-posterior projection). Severe regurgitation into the left ventricle is shown.

greatly improved after operation (Fig. 37, 38). His chest X-ray film showed considerable reduction in his cardio-thoracic ratio two months after operation (Fig. 36). Unfortunately, a diastolic murmur (grade 1/6) has been audible since three months postoperatively, but there has been no further progress in left ventricular hypertrophy according to electrocardiograms and no increase in the cardio-thoracic ratio on chest X-ray films.

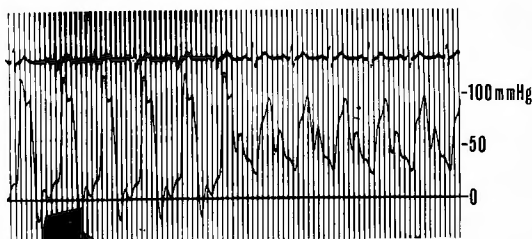
No diastolic murmur was audible in the other two successful cases, even eight months after insertion.



**Fig. 36** Chest X-ray film (postero-anterior projection) of case 4 two months after operation. Considerable reduction of the cardio-thoracic ratio has occurred, in comparison with that of Fig. 34.

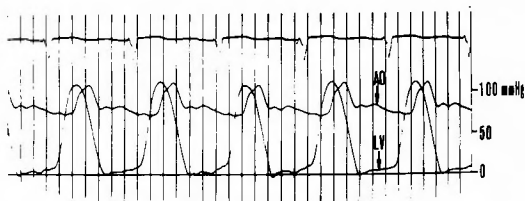
T.Y. 22y. ♂

LV AO



**Fig. 37** Withdrawal pressure recording from the left ventricle into the aorta before operation in case 4. Severe aortic incompetence and slight aortic stenosis are observed.

T.Y. 22y. ♂



**Fig. 38** Simultaneous pressure recording in the left ventricle and the aorta, immediately after homograft aortic valve replacement in case 4. Remarkable improvement is observed in the hemodynamics, as compared to the preoperative hemodynamics shown in Fig. 37.

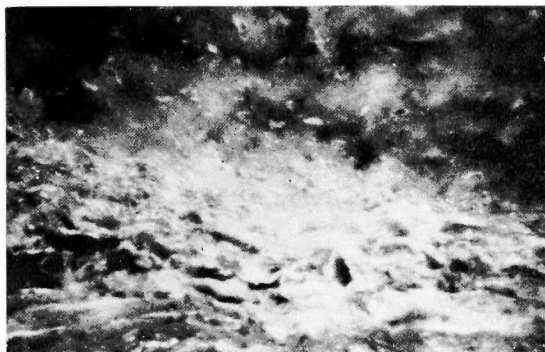
## F. Discussion

It is essential to determine when homografts should be excised from cadavers and which method of sterilization and preservation is the best.

Ross obtained them within 24 hours after death<sup>5)</sup>, and BARRATT-BOYES within 36 hours if the cadavers were refrigerated<sup>8)</sup>. HARRIS<sup>29)</sup> reported that aortic valves obtained 24 hours after death showed no reduction in tensile strength of the leaflet. The author, on the other hand, obtained homografts within 6 hours after death, on the basis of GROSS's report<sup>30)</sup>, and since in Japan cadavers are not ordinarily refrigerated.

There are several methods of sterilizing the grafts, such as immersion in 1% BPL<sup>26)</sup>, or 4% buffered acid formaldehyde<sup>31)</sup>, irradiation by electron beam<sup>29)32)33)</sup> and exposure

to ethylene oxide gas<sup>41</sup>). In order to minimize the reduction of tensile strength of the graft, irradiation by electron beam has recently been employed for sterilization. However, in spite of MEEKER's detailed experiments<sup>33</sup>) on sterilization of the aorta, it is essential to determine the strength of the electron beam adequate to completely sterilize the aortic valve without causing any serious histological changes after operation, since, in contrast to the



**Fig. 39** Collagen fibers on the ventricular side of a canine aortic valve which had been irradiated in the frozen state by electron beams at an energy level of 2.2 megarads in 14 seconds stained strongly with FITC-labeled anti-canine aortic valve rabbit antibody by fluorescent antibody technique.

aorta, homograft aortic valves consist of three different kinds of tissue: aortic cusp, cardiac muscle and aortic wall. In this study, sterilization by 1% BPL was used because of its strong sterilizing ability as well as its denaturation of antigenic substances in the graft. It was also demonstrated by the fluorescent antibody technique that electron beam irradiation to the canine aortic valve in the frozen state at an energy level of 2.0 to 2.2 megarads in short exposures of 13 to 14 seconds (Van de Graaff Electron Accelerator, Osaka Lab. for Rad. Chemist. Japan Atomic Energy Research Inst., Osaka, Japan) did not denature the antigenic substances in the valve (Fig. 39).

However, the author does not think that immersion in 1% BPL is the best method of sterilization of aortic valve homografts, since it greatly decreases the tensile strength of the valve<sup>29)32)</sup> and since cusp rupture after transplantation of BPL-treated homografts has been reported in clinical cases<sup>12)22)</sup>

There are several methods of preserving homografts, such as freeze-drying<sup>8)</sup>, freezing<sup>32)</sup> and immersion in a nutrient medium<sup>30)</sup>. A great reduction in tensile strength of the graft by the freeze-drying technique has been demonstrated<sup>29)</sup>, and storage in a nutrient medium for long periods is impossible. Therefore, a method of valve storage by freezing will be studied furthermore.

One of the reasons why homograft aortic valve replacement has not been widely accepted, is the rather high incidence of postoperative aortic insufficiency<sup>5)9)12)34)35)36)37)</sup>. In this study, postoperative aortic incompetence occurred in only one case, in the third postoperative month. It is expected that the incidence of insufficiency will decrease as surgical teams gain experience, as discussed in BIGELOW's report<sup>37)</sup>. However, during transplantation, special care should be taken at certain points: (1) The graft should have an inside diameter exactly 2 mm less than the recipient's aortic root. (2) Aortic root tailoring should be added when it is necessary to reduce the size of the host aorta. (3) The sutures at both the upper and the lower line of the graft should be placed close to each other in order to prevent peripheral suture line leakage. (4) Vertical mattress sutures should be placed below each commissure.

#### G. Summary

1. Aortic valve replacements with BPL-treated homografts were performed in four clinical cases, after the thorough fundamental experiments described in Parts I and II.

2. These operation were performed mostly by the method of BARRATT-BOYES, except that the lower line of the graft was sutured with interrupted mattress sutures.

3. One patient died on the third postoperative day with acute renal insufficiency and right ventricular failure.

4. The other three patients had uneventful postoperative courses and were discharged in excellent condition.

5. All three survivors are greatly improved. The follow-up period has been 6 to 8 months.

6. In only one of three patients, a postoperative diastolic murmur of aortic insufficiency became audible three months after operation, without any peripheral signs of aortic incompetence.

7. Some problems in regard to methods of sterilization and preservation of the aortic valve homograft and operative techniques were discussed.

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(\* In Japanese)

## 和文抄録

# 同種大動脈弁移植の実験的研究とその臨床応用 および蛍光抗体法による犬および人 大動脈弁の抗原性に関する研究

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1956年 Murray が大動脈弁疾患患者の下行大動脈へ同種大動脈弁の移植に成功し、1962年には Ross あるいは Barratt-Boyes が冠動脈下への同所性移植に成功して以来、次第に大動脈弁疾患の弁置換に際し、人工弁にかわつて同種弁が使用されるようになって来ている。同種弁が人工弁に比較してすぐれている点は、血行力学的に中心流を障害しないこと、術後血栓形成がほとんどなく抗凝固剤の投与が不要なること、術後感染が少いこと、などである。

著者は蛍光抗体法により大動脈弁自体の抗原性の追求をおこなうとともに、犬を使用して移植実験をおこない、その安全性を確認したのち臨床に応用した。

第1章: 犬大動脈弁の抗原性については犬大動脈弁の Emulsion の免疫電気泳動、および Ouchterlony 寒天板による抗犬大動脈弁家兔血清を犬血清によつて吸収する吸収試験、さらに新鮮犬大動脈弁を蛍光色素をラベルした抗犬大動脈弁家兔抗体、抗犬全血漿家兔抗体による2重染色によつて、犬大動脈弁には特有の組織抗原はなく、ただ犬血清蛋白成分の一部が抗原として存在することを証明した。これら抗原性を有する物質の犬大動脈弁における存在部位は蛍光抗体法によると、おもに弁葉の左心室側の弾性線維間に存在する膠原線維にあることを確認した。人犬大動脈弁については gamma Globulin の存在は証明出来たが Albumin については確認しえなかつた。

第2章: 犬を使用しての移植実験では同種大動脈弁の一弁を肺動脈弁部位および同所性に冠動脈下に移植する2群の移植実験を犬についておこなつた。またそれぞれの群において新鮮弁、1% beta-propiolactone滅菌、Hanks 氏液保存弁、凍結乾燥弁の3種類の同種弁を移植した。結果は肺動脈弁部位に移植した同種大動脈弁は3種類の弁すべてが術後3ヵ月ですでに肥厚、硬化し、その後時間の経過とともに石灰沈着をきたし、術

後2年でも rejection はされず、なおポケット状の形態は保つているが、完全に弁機能を失つていた。滅菌保存方法による差異としては beta-propiolactone 滅菌、あるいは凍結乾燥した同種弁の方が新鮮弁よりも肥厚、硬化、石灰化は軽度であつた。

これに反し冠動脈下にて同所性移植をおこなつた同種大動脈弁は、3種類の弁のすべてが萎縮、硬化をきたすことなく柔軟性を保ち完全に弁機能をはたしていた。最長観察例は術後2年である。滅菌保存方法の差異による組織学的な相違としては、処理弁の線維細胞の核の消失は新鮮弁に比して非常に早い事および移植弁と recipient の縫合部には新鮮弁では処理弁に比較してリンパ球、単核球、形質細胞の浸潤が強くみられた。しかし新鮮弁でも弁葉には免疫反応をおもわせる細胞浸潤はみられなかつた。宿主よりの組織の移植弁への侵入としては線維芽細胞の侵入および内皮細胞の被覆が証明され、たとえこれらの組織が移植弁に侵入して来たとしても同所性に移植された同種大動脈弁は柔軟性を保ち、弁機能をはたすことを確認した。

第3章: 以上の第1および第2章の基礎実験より大動脈弁には抗原性を有する組織の存在はみとめられるが、rejection はまったくなく、免疫反応も非常に軽度で、生着し、同所性に移植された場合にはこれが完全に機能をはたすことが確認されたので臨床に応用した。京都大学医学部附属病院第2外科において現在までに4例に応用した。移植弁の滅菌、保存方法、移植手技など各種検討の結果、世界的に一番多く行なわれている Barratt-Boyes の方法が安全、確実と思われたのでこれを採用した。結果は第1例を術後3日目に急性腎不全により失つたが、他の3例は人工弁移植に比し、やはり術直後よりきわめて良好なる血行動態を認めし、第2例は術後8ヵ月になるが経過はきわめて良好である。